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**INHIBITION OF GRANULOPOIESIS
IN DIFFUSION CHAMBERS BY
A GRANULOCYTE CHALONE**

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RECORD SET

Research was conducted according to the principles enunciated in the
"Guide for Laboratory Animal Facilities and Care," prepared by the
National Academy of Sciences - National Research Council.

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FOREWORD

(Nontechnical summary)

It has been postulated that white blood cell production may, in part, be controlled by low molecular weight inhibitory molecules called chalone. The chalone by definition is produced and released by mature white cells and, in turn, inhibits further production of new white cells in a reversible manner. Although this assumption was theoretically sound, its physiological significance in the production of white cells remained to be demonstrated.

The purpose of this investigation was to test the specific action of granulocyte chalone in a closed diffusion chamber culture system implanted in mice. The diffusion chamber system would provide a favorable but not autonomous internal milieu for white blood cell production which would proceed at a rate comparable to that found normally for stimulated growth in these animals.

The chambers are made with cell impermeable Millipore filters glued to a plastic ring. Chambers are sterilized, detoxified, filled with mouse bone marrow cells and then implanted intraperitoneally in host mice. Raw granulocyte chalone was obtained in the form of media conditioned for 20 hours by rat white cells ($2 \times 10^7/\text{ml}$) at 37°C . The raw white cell chalone was administered to host mice in 1.5-ml aliquots at 5, 20, 24, 28, 44 and 48 hours after chamber implantation.

Chamber cells harvested at daily intervals illustrated the significant inhibitory effect of chalone treatment on white blood cell production during the early phase of culture. The total number of chamber cells produced per stem cell was reduced by approximately 30 percent due to the inhibitory action of granulocyte chalone. The

reduction of granulocyte progeny may be the result of chalone acting during the early phase of culture to reduce the active growth fraction of the primitive cells from which white cells are derived (progenitor cells) and thereby diminishing the amplification potential inherent in the initial bone marrow cell inoculum.

ABSTRACT

Granulocyte chalone obtained from media conditioned by rat granulocytes was tested on normal and vinblastine treated regenerating mouse marrow placed in an in vivo diffusion chamber culture system. Raw granulocyte chalone when injected several times during the early phase of culture significantly altered the cellular growth pattern as well as the parent-progeny relationships of both normal and regenerating marrow established within the chamber milieu. The total number of chamber cells (granulocytes plus macrophages) produced per inoculated stem cell was reduced by approximately 30 percent due to the action of granulocyte chalone. The reduction of granulocyte progeny may be the result of chalone acting during the early phase of culture to reduce the active growth fraction of granulocyte progenitor cells and thereby diminishing the amplification potential inherent in the initial cell inoculum.

I. INTRODUCTION

Recent studies have indicated that granulopoiesis may be controlled in part by a feedback system of inhibitory regulators.^{4, 11, 15, 19, 20, 25-28} One such regulator may be a low molecular weight, dialyzable molecule found in certain animal sera and in media conditioned by normal and leukemic cells.^{18, 21, 25-27} Rytömaa and Kiviniemi^{26, 27} have suggested that this regulator may represent a tissue-specific class of inhibitor or granulocyte chalone after the concept introduced by Bullough and Laurence⁹ for the epidermal chalone. While the case for the epidermal chalone is most substantial, relatively few studies have dealt with the granulocyte chalone and specifically its regulatory role in in vivo granulopoiesis.

Rytömaa and Kiviniemi^{26, 27} as well as Paukovits²¹ have shown that chalones isolated from rat sera, calf sera and media conditioned by mature granulocytes will reduce the proliferation of granulocyte precursors in in vitro liquid culture of rat marrow cells in a tissue-specific and reversible manner. More recently, Benestad et al.⁴ have demonstrated that chalone isolated from rat granulocyte conditioned media can depress the cellular uptake of ³H-thymidine in granulocyte precursors proliferating in in vivo diffusion chambers. The same chalone treatment was found to have no effect on proliferating rat immunoblasts and macrophages, suggesting a tissue-specific inhibitory effect. Although these results indicated a reduced proliferation of precursor cells, the cell yield per chamber following chalone treatment was not reduced. It is possible that while chalone reduced the number of cells entering the DNA synthetic phase, the release from this inhibition resulted in a shortened cell cycle time and additional cell divisions, thereby compensating for any cellular loss due to treatment.

The present study was designed to examine the cell-specific action of a granulocyte chalone, obtained from the in vitro incubation of rat granulocytes, on the number of granulocyte progeny formed per inoculated progenitor cell in both normal and regenerating bone marrow implanted in in vivo diffusion chambers.

II. MATERIALS AND METHODS

Animals. Diffusion chamber recipients were male and female Swiss-Webster white mice, weighing 25-35 grams, from the Walter Reed Army Institute of Research colony. Eight- to twelve-week-old male and female B6D2F₁ hybrids (C57BL/6J x DBA/2J) supplied by Jackson Laboratory and bred at the AFRI were used as experimental animals. Sprague-Dawley male and female rats, weighing 180-220 grams, were used for the collection of peritoneal granulocytes and as marrow donors in the in vitro erythropoietin response test.

The in vivo colony forming assay was performed with syngeneic B6D2F₁ hybrid mice, male and female, 8-12 weeks old. They received a total of 900 rads in midline bilateral exposures from the AFRI ⁶⁰Co source at a dose rate of 153.8 rads/min.

Drugs. Diffusion chamber (DC) recipients were pretreated with an intravenous injection of 250 mg/kg cyclophosphamide (Cytosan, Mead Johnson) 24 hours prior to DC implantation.

Regenerating bone marrow was obtained from donor mice injected intraperitoneally with 4 mg/kg vinblastine (Velban, Eli Lilly and Company) 3 days prior to euthanasia for inoculation into DC's and implantation.

Granulocyte chalone. Control solutions used against the granulocyte chalone were nonpyrogenic saline and the crude aqueous extracts of rat spleen and thymus

obtained as described by Kiger.¹⁶ In essence, the organs were removed and finely chopped and then ground in 2 volumes of distilled water (final pH 6.5). After centrifuging at 20,000 x g for 1 hour, the pellet was discarded and the supernatant dialyzed against distilled water. After dialysis, the extract was sterilized by Millipore filtration and stored at -70°C until used. These solutions were injected in the same regimen and volumes as the granulocyte extract.

Diffusion chamber technique. Diffusion chambers were made from Millipore filters (Millipore Corporation, Bedford, Massachusetts), pore size 0.22 μ m, diameter 13 mm, glued to plastic rings. Each chamber was tested for leaks and sterilized by dry heat. Chambers were then detoxified according to the method described by Goodman et al.¹⁴ by placing them in Hanks' balanced salt solution (HBSS) with 5 percent horse serum plus antibiotics at 4°C for 24 hours followed by a change to fresh solution 24 hours prior to implantation.

Single cell marrow suspensions were prepared from the femora of several mice in the tissue culture media CMRL 1066 with 10 percent fetal calf serum (FCS) and were held at 4°C until chamber inoculation in 100- μ l volumes over the range of 3×10^4 to 5.4×10^5 cells/chamber. After inoculation each chamber was sealed with hot dental wax and held in CMRL 1066/10 percent FCS at 4°C until implantation. Usually two DC's were implanted per mouse under chloral hydrate or ether anesthesia. At various intervals, after implantation, host mice were euthanatized (cervical dislocation), DC's removed and shaken, unopened, in an enzyme solution (0.5 percent Pronase B, 5 percent Ficoll in basal medium Eagle pH 7.2) as described by Benestad² at room temperature for 50 minutes. The DC's were then transferred to spinner modified

HBSS/10 percent FCS and held at 4°C until opened. This treatment dissolves the cellular clots within the DC's and leaves a viable cell suspension for harvesting and further application. The chambers were each washed three times with 100 µl of spinner modified HBSS/10 percent FCS. Cell suspensions were dispersed and aliquots taken for smears and hemacytometer counting.

In vitro semisolid agar colony forming assay. This technique is as described by Worton et al.³¹ and Austin et al.¹ with the substitution of a single layer of agar for methylcellulose as the supporting medium. CMRL 1066/10 percent FCS was made up double strength with 10 percent trypticase soy broth and 5 percent horse serum and was supplemented with 30 µg/ml of L-asparagine and antibiotics. The cells were cultured at a concentration of 10^5 /dish in 1-ml volume of 2 x medium and previously boiled 0.6 percent agar (Difco, Detroit, Michigan) mixed 1:1 just prior to plating. Mouse fibroblast (L-929 cell) conditioned medium at 10 percent of culture volume plus 2 percent deionized bovine serum albumin was used as the colony stimulating factor. Colonies of more than 50 cells were counted after 7 days of incubation.

Granulocyte chalone was added in several concentrations at the time of plating and assayed for its capacity to inhibit colony growth at the end of the 7-day period. The inhibitory effect of chalone was also assayed against colony stimulating factor (CSF) obtained from the serum of endotoxin treated rats and mice. Salmonella typhosa endotoxin was injected intraperitoneally into mice (50 µg/.1 ml)²² and rats (200 µg/1 ml) and 2 hours later the animals were bled by cardiac puncture under ether anesthesia. The serum was dialyzed for 3 days against distilled H₂O and stored at -70°C until used. Chalone activity was always assayed against the amount of CSF which exhibited maximum stimulating activity.

Proliferation of rat marrow cells in liquid culture. Single cell suspensions of rat marrow cells (10^6 /ml) in HBSS/20 percent FCS were pipetted into petri dishes (60 x 16 mm) to a total 5-ml volume. Granulocyte chalone was added to triplicate cultures in concentrations from 0 to 50 percent of total culture volume. The control cultures received nonpyrogenic saline. ^3H -thymidine (^3H -TdR) (Schwarz-Mann, specific activity 6.0 Ci/mM) was added to each culture dish to a final concentration of 1 μCi /ml. These cultures were incubated at 37°C in a humidified CO_2 atmosphere for 6 hours. The total ^3H -TdR incorporated was determined by liquid scintillation counting of the previously twice washed trichloroacetic acid (TCA) precipitable material obtained from each culture.

Lymphocyte response to PHA. Single cell mouse spleen suspensions were brought to a concentration of 2×10^6 nucleated cells/ml in CMRL 1066/10 percent FCS. Three-milliliter aliquots were dispensed to sterile glass round bottom centrifuge tubes with 0.02 ml of phytohemagglutinin (PHA_p) (Difco) added to each experimental tube. Granulocyte chalone was added in several concentrations and assayed for its capacity to inhibit the lymphocytic blastogenic response. Cells were incubated at 37°C with a 10 percent CO_2 humidified atmosphere. Forty-two hours later and six hours before harvest, 2.5 μCi of ^3H -TdR were added to each tube. Cellular harvest was performed at 4°C , and the radioactivity in each cell pellet was subsequently determined in the 5 percent TCA precipitable material obtained after two successive washes at 4°C .

Marrow response to erythropoietin in vitro. Single cell rat bone marrow suspensions were brought to a concentration of 10^6 nucleated cells per ml in CMRL 1066/10 percent FCS and held at 4°C . Step 3 erythropoietin (Connaught Medical Research

Laboratories, Toronto, Canada, specific activity 2-15 units/mg protein) was dissolved in CMRL 1066/50 percent FCS at a concentration of 10 units/ml and stored at -70°C .

^{59}Fe citrate (New England Nuclear Corporation, Boston, Massachusetts) was obtained at a concentration of $10\text{ }\mu\text{g/ml}$ with specific activity of 10 mCi/mg and preincubated with autologous rat serum as a source of transferrin.^{17, 29} Erythropoietin was added to 5-ml cultures (60 x 16 mm petri dish) of 10^6 nucleated cells/ml to provide a fixed concentration of 0.25 units/ml of culture. ^{59}Fe , at a concentration of $10\text{ }\mu\text{Ci/ml}$ in CMRL 1066/50 percent FCS, was added to each culture dish in a volume of 0.1 ml and further incubated for 12 hours, after which the cells were harvested and washed twice prior to determining the ^{59}Fe uptake in the cell pellet. Granulocytic chalone was added to the culture plates in several concentrations at initiation of culture and assayed for its capacity to inhibit the in vitro erythrocytic response to erythropoietin.

Mouse fibroblast cultures. Single cell suspensions of mouse L-929 cells ($10^5/\text{ml}$) were inoculated into flasks (Falcon Plastic, 75 cm^2 and 25 cm^2 area) in Eagle's MEM/10 percent FCS. After 24 hours' incubation the medium was changed and adhering cells washed and refed with fresh medium containing 20 percent granulocyte extract. Forty-eight hours after inoculation and 3 hours before harvest, $2\text{ }\mu\text{Ci}$ of $^3\text{H-TdR}$ were added to each flask. Following incubation, the adhering cells were washed twice with cold (4°C) HBSS, trypsinized and harvested. Single cells were counted, viability assessed and then mixed with 5 percent TCA to obtain the acid precipitable material for radioactive counting. Results are expressed as cpm/ 10^6 cells. Viabilities and plating efficiencies were also determined after in vitro incubation of single cell suspensions with various chalone concentrations for 1 hour at 37°C .

Statistical methods. Differences between experimental means were tested with the Student's "t" test. Linear regression analysis, by the method of least squares, was used to describe the data on cells inoculated versus cells harvested. The significance of differences between the slopes and y-intercepts describing the data was determined using two-sided Student's "t" test.

III. RESULTS

Noncytotoxicity of granulocyte chalone preparation. To test the possibility of a cytotoxic effect of the granulocyte chalone preparation on sensitive hematopoietic progenitor cells, suspensions of normal and regenerating bone marrow were preincubated in Eagle's MEM/10 percent FCS with various concentrations of granulocyte chalone for 1 hour at 37⁰C. Following incubation, the cell suspensions were washed twice with, and resuspended in, Eagle's MEM/10 percent FCS and their viability determined with trypan blue prior to assay for in vivo and in vitro colony forming cells as well as cell growth in diffusion chambers.

Viability of the total marrow suspension as well as the incidence of various progenitor or stem cells assayed was not reduced by treatment with the raw chalone in varying concentrations up to 50 percent of the culture medium (Table I).

In vitro assays of granulocyte chalone specificity and noncytotoxicity.

³H-TdR uptake by rat marrow cells. The inhibitory activity of chalone was initially assayed by its ability to reduce the incorporation of ³H-TdR into rat bone marrow cells in vitro. The results indicated that the maximum inhibition of DNA synthesis was 35.2 percent with an average of 32 percent occurring over a range of chalone concentration from 20 to 50 percent (v/v) (Table II). Increases in chalone concentration

Table I. Effect of Preincubation with Granulocyte Chalone on the Incidence of Progenitor Cells in Mouse Marrow

Chalone* (percent)	Viability (percent)	Assay	Mean number of colonies (\pm S.D.)		
			A	B	C
0	88.7	CFC [†]	61 \pm 7	147 \pm 6	139 \pm 11
20	91.8	CFC	59 \pm 8	150 \pm 9	133 \pm 7
0	88.9	CFU [‡]	3.2 \pm 0.9		
20	88.2	CFU	3.4 \pm 1.2		
30	91.4	CFU	4.1 \pm 1.1		
50	86.3	CFU	3.1 \pm 0.7		
0	91.3	CFU _R [§]	7.0 \pm 2.0		
20	88.7	CFU _R	7.1 \pm 2.4		
0	83.8	DCPC ^{**}	7.69 $\times 10^5 \pm 0.46$		
50	86.4	DCPC	7.62 $\times 10^5 \pm 0.56$		
0	76.5	DCPC _R ^{††}	9.13 $\times 10^5 \pm 0.57$		
50	74.7	DCPC _R	9.09 $\times 10^5 \pm 0.71$		

* 0 percent chalone = equivalent amount (v/v) of nonpyrogenic saline

† CFC, in vitro agar colony forming cell assayed with: A. Mouse endotoxin sera, B. L cell CM, and C. Rat endotoxin sera. Colonies per 10^5 cells.

‡ CFU, in vivo spleen colony forming unit. Colonies per 10^4 marrow cells.

§ CFU_R, regenerating marrow as source of cells

**DCPC, in vivo diffusion chamber progenitor cell. Relative number indicated by mean chamber cellularity at day 4 of harvest.

††DCPC_R, regenerating marrow as source of cells for chamber inoculation

‡‡No significant difference within groups due to chalone treatment

Table II. Effect of Increasing Chalone Concentration on ³H-Thymidine Uptake by Rat Marrow in Liquid Culture

Chalone concentration (percent v/v)	Mean cpm per culture [†] (\pm S.D.)	Percent inhibition
0*	39,650 \pm 408	Control
10	35,232 \pm 182	11.3 p < .001
20	27,454 \pm 320	30.7 p < .0005
30	27,953 \pm 510	29.5
40	26,682 \pm 250	32.7
50	25,639 \pm 270	35.2

* Control values are means (\pm S.D.) of cultures containing equivalent amount of nonpyrogenic saline

† 5-ml rat marrow cultures at 10^6 cells/ml

beyond 20 percent did not significantly further reduce the incorporation of ^3H -TdR into proliferating cells.

In vitro colony forming assay. The granulocyte chalone was subsequently assayed for its capacity to block the action of colony stimulating factors from three different sources in the in vitro agar system using mouse marrow target cells. Table III shows that the presence of rat granulocyte chalone (10 percent v/v) caused a 50 to 71 percent reduction in the total number of colonies formed.

Table III. Effect of Granulocyte Chalone on Agar Colony Formation

CSF source†	Mean colony number*		Percent inhibition
	Control CSF	CSF + chalone‡	
Rat endotoxin serum	123 \pm 9	47 \pm 6	63.3
Mouse endotoxin serum	63 \pm 5	20 \pm 5	70.5
Mouse L cell CM	167 \pm 11	84 \pm 7	49.7

* Colonies per 10^5 mouse marrow cells (\pm S.D.) average of five replicate experiments

† CSF preparation, see Methods

‡ Chalone at a concentration of 10 percent (v/v) of culture medium

Lymphocyte response to PHA, rat marrow response to erythropoietin, proliferation of mouse fibroblasts. On the other hand, Table IV shows that the granulocyte chalone at a concentration of 20 percent of culture volume did not significantly reduce the lymphocytic response to PHAp, using both rat and mouse spleen cell suspensions and the fasted rat marrow response to erythropoietin, as measured by total ^3H -TdR and ^{59}Fe cellular uptake respectively. In addition, chalone did not

significantly affect viability, plating efficiency or ^3H -TdR uptake of proliferating mouse L-929 cells in monolayer culture (Table IV).

Table IV. Effect of Chalone on the Lymphocytic Response to PHA, Rat Marrow Response to Erythropoietin, and Proliferation of Mouse L Cells in Vitro

	Mean cpm per culture* (\pm S. D.)		
Rat spleen + Saline	22,790 \pm 758	NSD†	
Rat spleen + Chalone	23,410 \pm 710		
Rat spleen + PHA + Saline	77,900 \pm 1,195	NSD	
Rat spleen + PHA + Chalone	76,260 \pm 2,115		
Mouse spleen + Saline	654 \pm 48	NSD	
Mouse spleen + Chalone	644 \pm 51		
Mouse spleen + PHA + Saline	6,331 \pm 723	NSD	
Mouse spleen + PHA + Chalone	6,451 \pm 825		
Rat b.m. + Saline	1,811 \pm 494	NSD	
Rat b.m. + Chalone	1,695 \pm 287		
Rat b.m. + Ep. + Saline	3,932 \pm 272	NSD	
Rat b.m. + Ep. + Chalone	3,703 \pm 216		
Mouse L cells + Saline	30,458 \pm 1,208	$p < .05$	
Mouse L cells + Chalone	32,614 \pm 2,516		

* Mean values of ^3H -TdR uptake by rat (6×10^6) and mouse (6×10^6) spleen cells and mouse L cells (10^6) and ^{59}Fe uptake of rat bone marrow cells (5×10^6). Five replicate experiments.

† No significant difference due to chalone treatment

Effect of chalone on growth of mouse bone marrow cells implanted within diffusion chambers. Normal mouse bone marrow cells in diffusion chambers were implanted in host animals and subsequently subjected to a regimen of granulocyte chalone injections designed to alter the proliferative capacity of the granulocyte progenitor and precursor cells replicating during the period of initial chamber growth.

Normal marrow. Figure 1 shows that granulocyte chalone significantly depressed growth of cells in the chambers when injected intraperitoneally in 1.5-ml aliquots as early as 5 hours after implantation and thereafter at 20, 24 and 28 hours during the 1st day and at 44 and 48 hours during the 2nd day of culture. Chamber growth in normal nonregenerating marrow was depressed throughout the period of treatment to approximately 58 percent of the initial inoculum at day 2 of culture, while control treated cultures had already initiated exponential growth beyond day 1.

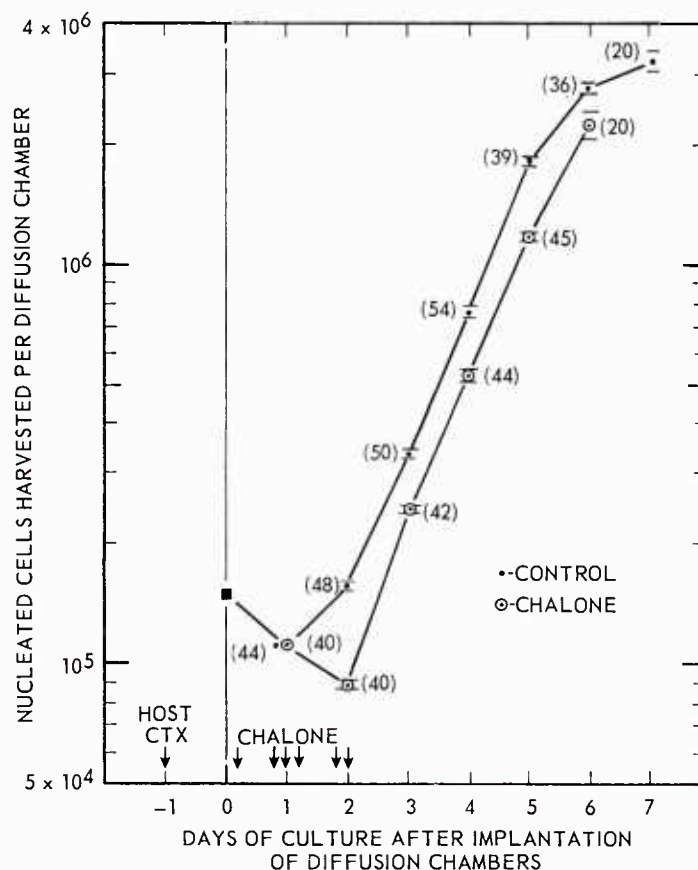


Figure 1. The effect of chalone injections on the growth of cells (granulocytes and macrophages) in diffusion chambers inoculated with 1.5×10^5 normal bone marrow cells. Results of five replicate experiments. Number of chambers harvested for each mean value (\pm SEM) indicated in parentheses.

Cessation of chalone injections resulted in a phase of rapid growth and cell recovery to values within 70 percent of control by the 3rd day of culture. During this recovery or rebound phase, the chalone treated cells increased 2.7-fold with a doubling time of 16.8 hours while control treated cells had only increased 2.2-fold, doubling every 20.8 hours. Thereafter, from days 3 through 5, the growth rates were similar for both groups. However, the average cellularity (proliferative and nonproliferative granulocytes and macrophages) through day 6 of culture remained significantly lower for chalone treated chambers, as compared with controls ($p < 0.001$).

In addition, it was noted that single, daily 1.5-ml injections of the chalone preparation had no effect on chamber cellularity when injected from days 1 through 4 after implantation.

Regenerating marrow. Figure 2 shows the similar pattern of inhibition and recovery observed with chalone treated regenerating marrow. Note that regenerating marrow in diffusion chambers showed an immediate exponential growth without the lag period seen with normal marrow. Chalone injected over the same regimen as with normal marrow slowed the initial 24-hour rapid growth phase (14.4 hours to 20.6 hours doubling time) seen with control treated cultures and resulted in a marked 48 percent deficit in total cellularity at day 2 of culture. Upon cessation of chalone treatment, this cellular deficit was diminished by a rebound phase with an approximate 16-hour doubling time during the subsequent 24-hour period, as compared with a 22-hour value for the control treated cells.

Effect of granulocyte chalone on chamber cellularity. The granulocyte chalone, as administered in our system, reduced the quantitative but not linear relationship

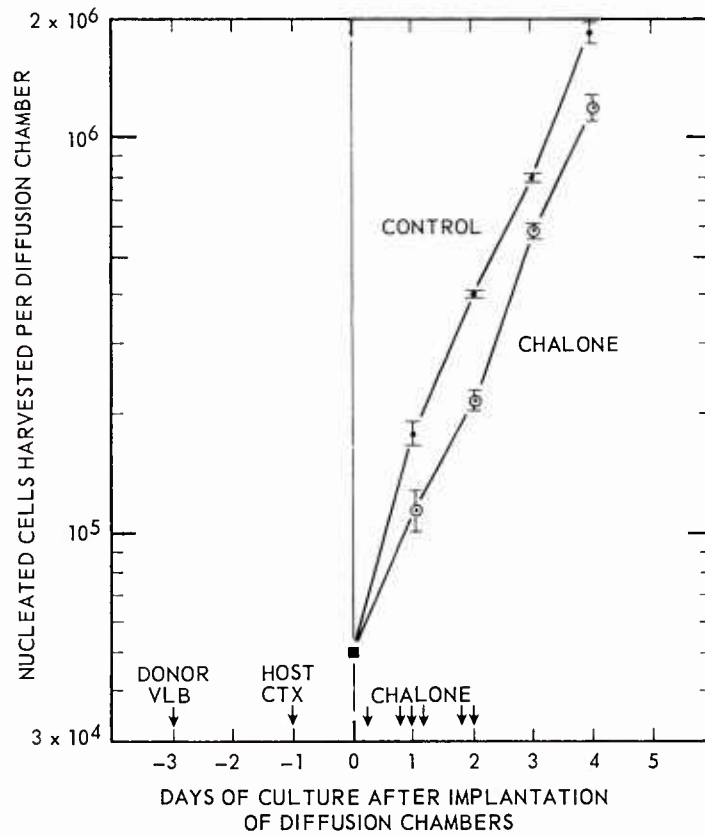


Figure 2. The effect of chalone on the growth of granulocytes and macrophages in diffusion chambers inoculated with 5×10^4 regenerating bone marrow cells. Results of a duplicate experiment with a total of 20 chambers for each mean value (\pm SEM). See text for pretreatment.

established within the milieu of the control treated chambers between the size of the marrow inoculum (and therefore progenitor cell content) and the number of granulocyte and macrophage progeny formed by day 4 of culture over the range of inoculum size from 3×10^4 to 5.4×10^5 cells. Statistical comparison of the linear regression lines in Figure 3 showed that while chalone treatment did not significantly affect the linearity within marrow groups (normal and regenerating) the amount of granulocytic

progeny formed was indeed significantly reduced in both marrow groups over the cell dose range studied ($p < 0.001$).

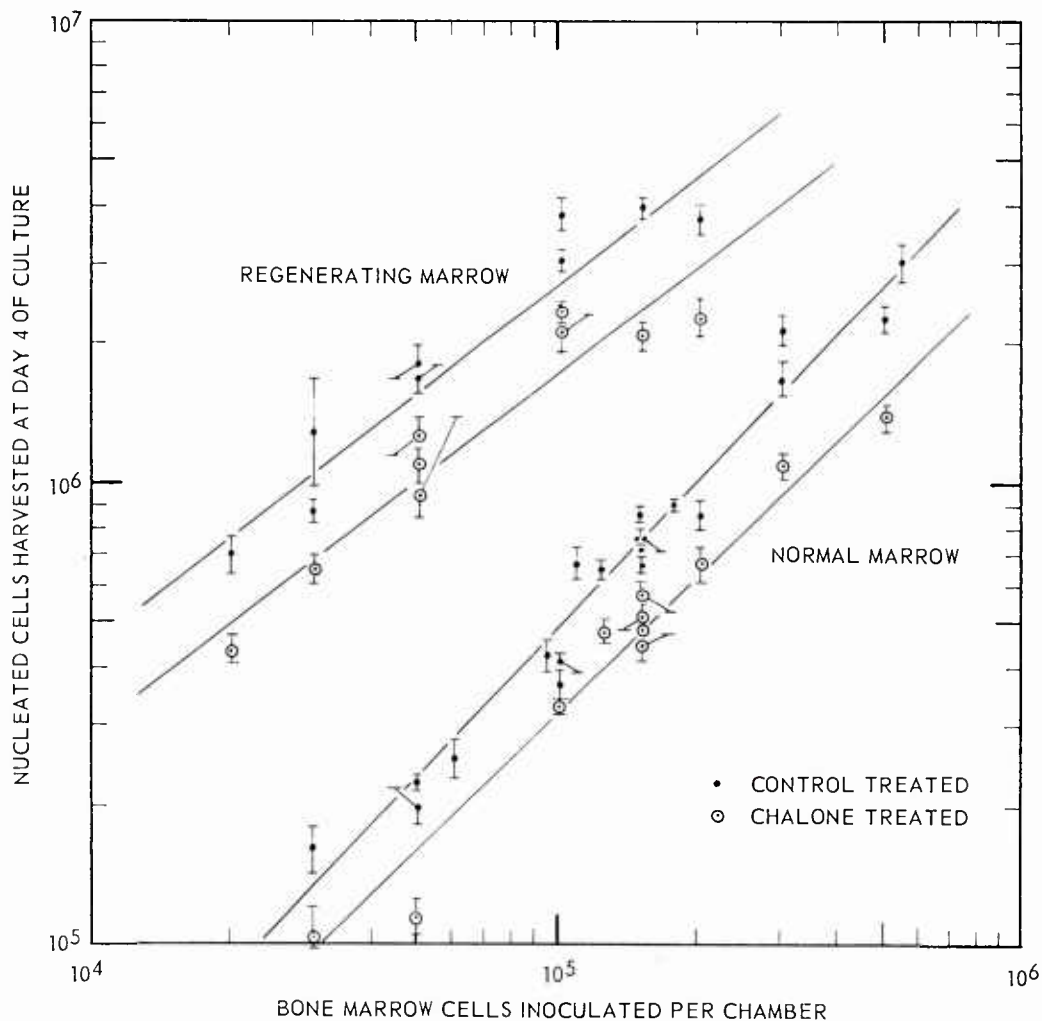


Figure 3. The number of bone marrow cells inoculated, normal and regenerating, versus the mean number of granulocytes plus macrophages harvested at day 4 of culture (\pm SEM). The regression lines for regenerating marrow are described by the equations $y = (27.099) x^{0.786}$ and $y = (17.299) x^{0.773}$ for control and chalone treated groups respectively (respective correlation coefficients 0.913 and 0.880). Normal marrow relationships are described by $y = (4.703) x^{1.070}$ and $y = (3.123) x^{1.041}$ for control and chalone treated groups respectively (respective correlation coefficients 0.963 and 0.950).

Table V shows the reduced number of granulocytic (proliferative and nonproliferative) progeny formed both per stem cell (CFU) and marrow cell inoculated as a result of chalone treatment of both normal and regenerating marrow. Chalone reduced the number of progeny formed at day 4 of culture by approximately 30 percent in both cases. The number of granulocytes formed per CFU with normal marrow inocula was reduced from 12.6×10^3 to 8.6×10^3 while for regenerating marrow the values decreased from 35.3×10^3 to 22.8×10^3 .

Table V. Effect of Chalone on the Number of Granulocyte Progeny Formed per Stem Cell (CFU) and per Marrow Cell Inoculated

Marrow inoculum	Treatment	Granulocyte progeny* per CFU inoculated	Percent reduction	Granulocyte progeny per marrow cell inoculated
Normal	Control	12.6×10^3	30.0	4.2
	Chalone	8.6×10^3		2.8
Regenerating	Control	35.3×10^3	30.0	25
	Chalone	22.8×10^3		16

* Granulocytes (proliferative plus nonproliferative) harvested at day 4 of culture

IV. DISCUSSION

The results of our experiments indicated the presence of an inhibitor or chalone in the granulocyte conditioned medium which exerted a tissue-specific, noncytotoxic and species nonspecific inhibitory effect on both in vitro and in vivo granulopoiesis in diffusion chamber cultures.

The granulocyte chalone, by definition, should inhibit only granulocytic precursor cells from entering the DNA synthetic phase while proliferation in other hematopoietic cell lines continues. Rytömaa and Kiviniemi^{26,27} using ^3H -TdR uptake in

combination with autoradiography of rat marrow cells in liquid culture have shown that an inhibitor present in their granulocyte extract can selectively depress the proliferation of granulocyte precursor cells while leaving unaffected the remaining proliferative cell types. Paukovits²¹ has reported on a chalone isolated from calf serum which also selectively reduced proliferation of granulocytic cells in normal marrow culture. Our initial in vitro assay of the chalone preparation showed a similar partial inhibition of total DNA synthesis in normal marrow. Furthermore, in vitro experiments clearly showed that the chalone preparation had no significant effect on the splenic response to PHA, the rat marrow response to erythropoietin or the proliferation of mouse L-929 cells in monolayer culture. A large inhibitory effect on these cellular responses would have indicated the presence of indiscriminate toxins or inhibitors leading to reduced proliferation of all cell types. Also, short-term preincubation of marrow suspensions with the chalone preparation failed to reduce the number of sensitive stem and progenitor cells. The same granulocyte extract, however, markedly reduced the formation of in vitro agar granulocytic colonies.

This result is consistent with the results reported by others^{11,12,15,18-20,28} describing the action of both dialyzable and nondialyzable molecules found in some sera and in media conditioned by spleen, bone marrow, granulocyte and leukemic cells capable of blocking the in vitro action of CSF in agar culture.

While these studies have demonstrated the in vitro, tissue-specific action of the inhibitory factors, their role in in vivo granulopoiesis remains to be demonstrated.

Our subsequent experiments were designed to test the tissue-specific action of granulocyte chalone in diffusion chamber culture, a closed cellular system which

provides a favorable, but not autonomous, in vivo milieu for myelopoiesis. Our results demonstrated that granulocyte chalone, when repeatedly injected during the early phase of culture, altered the cellular growth pattern as well as the parent-progeny relationship established within the chamber environment with both normal and regenerating marrow inocula. The effectiveness of the granulocytic chalone when administered during the early phase of culture was most likely dependent on its ability to reduce the active growth fraction of granulocytic progenitor and precursor cells relative to control cultures, thereby diminishing the amplification potential inherent in the initial cell inoculum.

Benestad et al.⁴ have recently used the diffusion chamber system to illustrate the in vivo inhibitory action of a chalone preparation on proliferating granulocyte precursors 5 days after the initiation of chamber culture. Their granulocyte extract, when injected two to three times into the host mice, significantly reduced ³H-TdR uptake into proliferating granulocytes while having no effect on proliferating immunoblasts and macrophages. Although their data indicated a reduced proliferation of the granulocytic precursor pool, there was no reduction in granulocyte yield following chalone treatment. It is possible that the reduced proliferation was compensated for by shortened cell cycle parameters and additional divisions following release from the inhibitory effect of the chalone; whereas, in our system, the compensatory proliferation following cessation of chalone treatment could not equal the cellular production of control chambers because chalone, injected during the early phase of culture, inhibited the growth of the progenitor pool at a time when its proliferative potential was being established within the chamber milieu.

The importance of events taking place during the initial 48 hours of culture has been well documented.^{3,5-8} Breivik et al.,⁸ using chamber to chamber transfers in nonpretreated hosts, have illustrated the "rapid increase of functioning progenitor cells between the first and second day of culturing." The reduction of this effect through the use of hydroxyurea confirmed the increased proliferative state of the progenitor cells. In addition, Benestad³ has shown through the use of cell cycle specific drugs that a significant number of chamber cells in nonpretreated hosts had entered the S-phase within 18 hours of implantation.

Boyum et al.⁶ using preirradiation of chamber hosts in combination with transfer of chambers to nonpretreated hosts have shown significantly enhanced granulopoiesis in response to increased stem and progenitor cell self-renewal during the early phase of culture. Tyler et al.³⁰ have also shown a sustained differential growth of myeloid elements as well as stem cells in neutropenic, Cytosan pretreated hosts.

These results and others^{23,24} suggested that the potential for proliferation and amplification of the stem and granulocyte progenitor cell populations is rapidly established within the early phase of chamber culture in a milieu conditioned by the presence of diffusible growth factors. It was reasoned, therefore, that the chalone would be most effective when administered during this phase of chamber culture.

In the present study, it was shown that the early administration of chalone reduced granulocyte progenitor cell self-replication and prevented an increase in the active growth fraction, which was necessary for full precursor amplification, under the differentiative stress established within the Cytosan pretreated hosts. It is also possible that a relative reduction in the active growth fraction could have occurred

through a premature differentiation of the chalone blocked progenitor cells. The retardation of division over a critical length of time may allow expression of tissue functional genes directing differentiation with subsequent loss of amplification inherent in the system.^{10,13}

REFERENCES

1. Austin, P. E., McCulloch, E. A. and Till, J. E. Characterization of the factor in L-cell conditioned medium capable of stimulating colony formation by mouse marrow cells in culture. *J. Cell. Physiol.* 77:121-133, 1971.
2. Benestad, H. B. Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leukocytes. *Scand. J. Haematol.* 7:279-288, 1970.
3. Benestad, H. B. Cell kinetics in diffusion chambers: survival, resumption of proliferation, and maturation rate of murine haemopoietic cells. *Cell Tissue Kinet.* 5:421-431, 1972.
4. Benestad, H. B., Rytömaa, T. and Kiviniemi, K. The cell specific effect of the granulocyte chalone demonstrated with the diffusion chamber technique. *Cell Tissue Kinet.* 6:147-154, 1973.
5. Bøyum, A. and Borgstrøm, R. The concentration of granulocytic stem cells in mouse bone marrow, determined with diffusion chamber technique. *Scand. J. Haematol.* 7:294-303, 1970.
6. Boyum, A., Carsten, A. L., Laerum, O. D. and Cronkite, E. P. Kinetics of cell proliferation of murine bone marrow cells cultured in diffusion chambers: effect of hypoxia, bleeding, erythropoietin injections, polycythemia, and irradiation of the host. *Blood* 40:174-188, 1972.
7. Breivik, H. and Benestad, H. B. Regulation of granulocyte and macrophage formation in diffusion chamber cultures of mouse haematopoietic cells. *Exptl. Cell Res.* 70:340-348, 1972.
8. Breivik, H., Benestad, H. B. and Bøyum, A. Diffusion chamber and spleen colony assay of murine haematopoietic stem cells. *J. Cell. Physiol.* 78:65-72, 1971.
9. Bullough, W. S. and Laurence, E. B. Mitotic control by internal secretion: the role of the chalone-adrenalin complex. *Exptl. Cell Res.* 33:176-194, 1964.
10. Bullough, W. S. and Rytömaa, T. Mitotic homeostasis. *Nature* 205:573-578, 1965.
11. Chan, S. H. Influence of serum inhibitors on colony development in vitro by bone marrow cells. *Australian J. Exptl. Biol. Med. Sci.* 49:553-564, 1971.
12. Chan, S. H., Metcalf, D. and Stanley, E. R. Stimulation and inhibition by normal human serum of colony formation in vitro by bone marrow cells. *Brit. J. Haematol.* 20:329-341, 1971.

13. Frankfurt, O. S. Epidermal chalone. Effect on cell cycle and on development of hyperplasia. *Exptl. Cell Res.* 64:140-144, 1971.
14. Goodman, S. A., Chen, M. G. and Makinodan, T. An improved primary response from mouse spleen cells cultured in vivo in diffusion chambers. *J. Immunology* 108:1387-1399, 1972.
15. Ichikawa, Y., Pluznik, D. H. and Sachs, L. Feedback inhibition of the development of macrophage and granulocyte colonies. I. Inhibition by macrophages. *Proc. Natl. Acad. Sci.* 58:1480-1486, 1967.
16. Kiger, N. Isolation and immunological study of thymic lymphocytic inhibitory factors. *Rev. Europ. Études Clin. et Biol.* 16:566-572, 1971.
17. McCool, D., Miller, R. J., Painter, R. H. and Bruce, W. R. Erythropoietin sensitivity of rat bone marrow cells separated by velocity sedimentation. *Cell Tissue Kinet.* 3:55-65, 1970.
18. Metcalf, D. Inhibition of bone marrow colony formation in vitro by dialysable products of normal and neoplastic hematopoietic cells. *Australian J. Exptl. Biol. Med. Sci.* 49:351-363, 1971.
19. Niskanen, E., Quesenberry, P., Stohlman, F., Jr., Levin, J. and Ryan, M. Colony-stimulating and inhibiting factors in sera from normal and endotoxin-treated CF₁ mice. *Proc. Soc. Exptl. Biol. Med.* 143:176-180, 1970.
20. Paran, M., Ichikawa, Y. and Sachs, L. Feedback inhibition of the development of macrophage and granulocyte colonies. II. Inhibition by granulocytes. *Proc. Natl. Acad. Sci.* 62:81-87, 1969.
21. Paukovits, W. R. Control of granulocyte production: separation and chemical identification of a specific inhibitor (chalone). *Cell Tissue Kinet.* 4:539-547, 1971.
22. Quesenberry, P., Morley, A., Stohlman, F., Jr., Rickard, K., Howard, D. and Smith, M. Effect of endotoxin on granulopoiesis and colony-stimulating factor. *New Engl. J. Med.* 286:227-232, 1972.
23. Rothstein, G., Hügl, E. H. and Athens, J. W. The relation of diffusible granulocytopoietic stimulator (DGS) to neutropenia and neutrophil releasing factor (NRF). *Blood* 38:820 (Abstract 100), 1971.
24. Rothstein, G., Hügl, E. H., Chervenick, P. A., Athens, J. W. and Macfarlane, J. Humoral stimulators of granulocyte production. *Blood* 41:73-78, 1973.

25. Rytömaa, T. Role of chalone in granulopoiesis. *Brit. J. Haematol.* 24:141-146, 1973.
26. Rytömaa, T. and Kiviniemi, K. Control of granulocyte production. I. Chalone and antichalone, two specific humoral regulators. *Cell Tissue Kinet.* 1:329-340, 1968.
27. Rytömaa, T. and Kiviniemi, K. Control of granulocyte production. II. Mode of action of chalone and antichalone. *Cell Tissue Kinet.* 1:341-350, 1968.
28. Shadduck, R. K. Granulocyte stimulating and inhibiting activity from neutrophils (PMN's): possible dual feedback control of granulopoiesis. *Blood* 38:820 (Abstract 101), 1971.
29. Stephenson, J. R. and Axelrad, A. A. Separation of erythropoietin-sensitive cells from hemopoietic spleen colony-forming stem cells of mouse fetal liver by unit gravity sedimentation. *Blood* 37:417-427, 1971.
30. Tyler, W. S., Niskanen, E., Stohlman, F., Jr., Keane, J. and Howard, D. The effect of neutropenia on myeloid growth and the stem cell in an in vivo culture system. *Blood* 40:634-645, 1972.
31. Worton, R. G., McCulloch, E. A. and Till, J. E. Physical separation of hemopoietic stem cells from cells forming colonies in culture. *J. Cell. Physiol.* 74:171-182, 1969.

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13. ABSTRACT Granulocyte chalone obtained from media conditioned by rat granulocytes was tested on normal and vinblastine treated regenerating mouse marrow placed in an <u>in vivo</u> diffusion chamber culture system. Raw granulocyte chalone when injected several times during the early phase of culture significantly altered the cellular growth pattern as well as the parent-progeny relationships of both normal and regenerating marrow established within the chamber milieu. The total number of chamber cells (granulocytes plus macrophages) produced per inoculated stem cell was reduced by approximately 30 percent due to the action of granulocyte chalone. The reduction of granulocyte progeny may be the result of chalone acting during the early phase of culture to reduce the active growth fraction of granulocyte progenitor cells and thereby diminishing the amplification potential inherent in the initial cell inoculum.			